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Industrial applications of enzymes have been limited by the availability of robust, reliable and economical protein-based catalysts The goal of the work outlined in this proposal was to continue development and commercialization of an iterative process to rapidly evolve sequence variants of selected enzymes with enhanced industrial potential. This directed evolution approach involves the use of random mutagenic techniques followed by activity screening of thousands of resulting mutant clones under selected assay conditions. Screening for activity in successively higher concentrations of a selected solvent following sequential rounds of mutagenesis results in the accumulation of amino

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Final Report: Directed Evolution Process for Robust Enzyme Catalysis in Organic Solvents

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Technical abstract

Industrial applications of enzymes have been limited by the availability of robust, reliable and economical protein-based catalysts The goal of the work outlined in this proposal was to continue development and commercialization of an iterative process to rapidly evolve sequence variants of selected enzymes with enhanced industrial potential. This directed evolution approach involves the use of random mutagenic techniques followed by activity screening of thousands of resulting mutant clones under selected assay conditions. Screening for activity in successively higher concentrations of a selected solvent following sequential rounds of mutagenesis results in the accumulation of amino acid substitutions which synergistically contribute to optimum activity. Phase I results of this work have shown that a percentage of clones expressing the serine protease subtilisin, when subjected to error-prone PCR mutagenesis, and subsequently screened for improved ligase activity in dimethylformamide (DMF), produced enzyme which catalyzed the polymerization of amino acids into extended peptides. Random PCR mutagenesis has been effectively applied to select clones of subtilisin which effectively catalyze the ligation of amino acids into polymeric peptides in high concentrations of DMF. Clones identified as having ligase activity in solvent were be sequenced to determine amino acid substitutions. The resultant enzymes can take advantage of the high enantio- and diastereoselectivity of enzyme-catalyzed synthesis while overcoming the drawbacks of low enzyme stability and reactivity in polar, non-aqueous solvent.

Anticipated benefits and commercial applications

The resulting economic benefits will include the rapid stereoselective synthesis of peptides with minimal by-product generation and low environmental impact. Commercial applications will include the preparation of novel materials such as chiral polymers, pharmaceuticals or agrochemicals containing natural or non-natural, D- or L-amino acids. These materials can be designed at the sequence level for folding into highly ordered α–helix or β-sheet structures. When crosslinked appropriately, these polymers will yield highly ordered tertiary structures which may, for example, be used for production of non-linear optic or nanotube materials.

Keywords: Biocatalysis, enzymes, subtilisin, polymer, ligase, chiral, enantioselectivity, protease

Project Summary

Significance

There is a crucial need in the chemical industry for efficient catalysts for the practical synthesis of optically pure materials. Classes of molecules and compounds which are key players in established and emerging markets must be provided by industry in economical and environmentally acceptable ways. The synthesis of polymers, pharmaceuticals, natural products and agrochemicals is often hampered by expensive technologies which produce harmful byproducts and which suffer from low enantioselectivity. Enzymes have a number of remarkable advantages which can overcome these problems in catalysis; they act on single functional groups, distinguish between functional groups on a single molecule and distinguish between enantiomers. Moreover, they are biodegradable and function at very low molar fraction of reactions. Because of their chemo-, regio- and stereospecificity, enzymes present a unique opportunity to optimally achieve desired selective transformations. Use of enzymes for technological applications, however, may require optimization of activities in environments or on substrates for which they were not evolutionarily selected. Enzymes have evolved by selective pressure to perform very specific biological functions within the milieu of a living organism. For the most part, the >3000 enzyme activities thus far described by modern biochemical science (1) have been isolated from organisms derived from a small fraction of the available phylogenetic diversity. These enzymes have evolved to function within cells under conditions of mild temperature, pH and salt concentration.

The spectrum of environments in which enzymes function has been dramatically expanded by recent research aimed at increasing their stability and recovering their catalytic activities in environmental extremes once thought beyond the realm of biocatalysis. Using random mutagenesis to accelerate evolution, certain features of enzymes, including catalytic activity (2,3), thermostability (4-6), alkaline stability (7) and substrate specificity (8) have been enhanced.

Since many industrial reactions occur in organic solvents, potential uses of enzymes are limited by their insolubility and instability in these environments. It has been demonstrated that enzymes are able to substitute critical residues and optimize catalysis in polar organic solvents. Using random mutagenesis and screening, variants of the serine protease subtilisin have been selected which have specific amino acid sequence alterations and which catalyze the enzyme's peptide hydrolytic reaction in dimethyl formamide (DMF) at efficiencies approaching those of the wild-type enzyme in water. Furthermore, the evolved enzyme variants have been shown to catalyze transesterification reactions and the kinetically controlled synthesis of poly(L-methionine)(9,10) in DMF.

The overall goal of Phase I of this project was to direct the evolution of subtilisin to catalyze enantiospecific ligation of natural and non-natural amino acids in organic solvents to form peptide polymers. The approach aimed to select variants of subtilisin which catalyze ligase activity in DMF at efficiencies not possible with aqueous-based subtilisin. Further screening for stereospecificity and substrate selectivity in solvent will result in selection of enzyme variants with properties desirable for synthesis of complex polymers of defined sequence and chirality. Choice of monomer and monomer sequences will allow the formation of predictable highly ordered secondary and tertiary structures with highly desirable properties for materials, medical and agrochemical industries.

Progress report:

Objectives of Phase I

Develop an assay for amino acid ligation
Measure kinetics using assay
Perform rounds of random mutagenesis on subtilisin clone
Choose improved mutants

Sequence and identify mutations in improved clones
Isolate subtilisin with improved ligation ability at high concentrations of DMF

Subtilisin Background

Hydrolases represent one of six general classes of enzymes as defined by the International Union of Biochemistry and Molecular Biology (1). These enzymes catalyze the cleavage of C-O, C-N and C-C bonds. Proteases, a subclass of the hydrolases, act on peptide bonds, (i.e., amides) cleaving the C-N bond to yield the carboxylic acid and amine. Proteases are further subdivided into four subclasses based upon mechanism and active site residues: serine, cysteine, metallo- and aspartate proteases. The serine protease class of which subtilisin is a member, work via the formation of an acyl-serine intermediate, this serine being part of the serine-histidine-aspartate catalytic triad.

Subtilisin is a monomer of MW ~27kDa which is broadly specific for peptide substrates. It catalyzes hydrolysis of sequences containing a large, uncharged residue in the P1 position, ie the acyl donor group where peptide scission takes place. (11). The enzyme has been isolated from various species of the genus *Bacillus*. The single polypeptide chain varies in length in a species-dependent manner around 274 residues. (12-15). There are currently amino acid sequences available for at least 14 subtilisins (16). The sequences exhibit high homology which is especially evident around the primary and secondary substrate binding site, Ser^{125}-Leu^{126}-Gly^{127}-Gly^{128} and Tyr^{104} and around the serine nucleophile, Asn^{218}-Gly^{219}-Thr^{220}-**Ser^{221}**-Met^{222}-Ala^{223}. The catalytic triad consists of Asp^{32}, His^{64} and Ser^{221} and an oxyanion binding site is comprised of Asn^{155} and the main chain amide of Ser^{221}

More than 50 high resolution structures of subtilisin have been determined with substrates, transition state analogues and inhibitors bound to the active site or with various site-specific mutations (11). The enzyme exhibits typical Michaelis-Menten kinetics with synthetic peptide or natural protein substrates. Using structural information, kinetic analyses and mutagenesis, the mechanism for peptide bond hydrolysis now well understood.

Subtilisin-catalyzed organic synthesis

In addition to peptide bond hydrolysis, proteases are able to catalyze the hydrolysis of esters, since the first step of the half-reaction, formation of the acyl enzyme intermediate, works with either esters or amides. The second half of the reaction, cleavage of the acyl serine intermediate, can be viewed as a transfer of the acyl group to water. Other nucleophiles (specifically amines and alcohols) can intercept the acyl serine leading to the synthesis of amides and esters. Thus peptide, ester and amide hydrolysis or, upon reversal of the hydrolysis reaction, ester, amide and peptide bond formation, may be catalyzed by proteases. These characteristics along with exquisite stereoselectivity and ready availability have made these enzymes attractive catalysts for synthesis reactions involving hydrolysis, transesterification, transacylation and transamidation (for a review, see 17). Subtilisin has been used for regioselective ester hydrolysis of dibenzyl esters of aspartate and glutamate (18) and for regioselective hydrolysis of 1,2,3 propan-tricarboxylylic esters (19). Subtilisin has also been used for the regioselective acylation and deacylation of carbohydrates and carbohydrate derivatives (20,21), for the resolution of mixtures of racemic amines (22) and for the regioselective oxidation of steroids (23). It has also been employed in the biocatalytic synthesis of sucrose polymers (24) as well as for synthesis of optically active methacrylic polymers from a racemic ester mixture (25).

Protease-catalyzed peptide and polymer synthesis, ie amino acid ligase activity, has been investigated in recent years to attempt to overcome a number of disadvantages encountered with conventional chemical routes (26-28). Specifically, protease-based methods are regio- and stereoselective and are free of racemates. Thus extensive post-

synthesis purification steps associated with chemical peptide syntheses are unnecessary. Furthermore, the protease-catalyzed synthesis does not require the steps of sidechain protection and deprotection. The advantages and disadvantages of chemical and enzymatic peptide synthesis have been outlined by Faber (29).

Table 1: Pros and Cons of chemical and enzymatic peptide synthesis

	Chemical	Enzymatic
Stereoselectivity	low	high
Regioselectivity	low	high
Amino acid range	broad	limited
Protective grp requirements	high	low
Purity Reqs of starting Materials	high	low
By-products	some	negligible
Danger of racemization	some	none

From: Faber, K. "Biotransformations in Organic Chemistry", Springer NY 1992

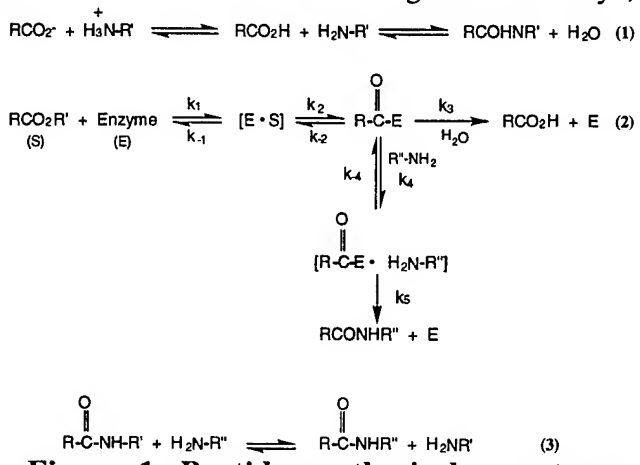


Figure 1. Peptide synthesis by proteases

There are three strategies for peptide or polymer synthesis by subtilisin and by proteases in general (Figure 1:ref 30). One is the thermodynamic method which takes advantage of the reversal of the hydrolysis reaction (**1**). The second is a kinetic method which involves aminolysis of N-protected esters (**2**). The second method is fast and has been shown to be effective for peptide synthesis (31). A third method is a transpeptidation reaction (**3**).

One disadvantage of protease-based peptide synthesis is that hydrolysis of the enzyme-bound acyl intermediate is favored over the desired aminolysis reaction. One first solution to this problem has been the construction of a site-directed double mutant of subtilisin which functions in water (32,33). The aminolysis/hydrolysis ratio has also been shifted by performing the reaction with native subtilisin suspended in organic solvent (34).

D. Directed evolution of subtilisin

To date, the synthesis reactions noted above have been of academic interest and the industrial uses of proteases have been confined to the detergent and food industries which take advantage of the enzyme's simple hydrolytic activity. The disadvantages of using proteases for amino-acid ligase-based peptide synthesis, ie, low solubility of the growing peptide and high aqueous substrate preference have also limited their use for polymer synthesis.

Using emerging techniques of molecular biology and specifically designed screening methods a first step has been taken toward the rapid evolution and selection of subtilisins which are effective catalysts for hydrolysis in non-aqueous environments

(9,10). The original goal of this research was to obtain proof that enzymes could function at wild-type/aqueous efficiencies in organic solvents.

Polar and non-polar organic solvents have dramatic effects on the turnover of subtilisins as well as of other enzymes. Polar and non-polar organic solvents can have substantial effects on the catalytic rate constant, kcat, or the Michaelis constant, Km, resulting in orders of magnitude effects on catalytic efficiency reflected in the ratio kcat/Km. Mutations which recover the wild-type values of these constants are therefore desirable, enabling the use of the enzyme in a target solvent.

1. Assay development

The first phase of the project was dedicated to the conceptualization and implementation of an effective assay for high volume screening of mutated clones. This assay had to be able to discern increases in the rate of peptide formation over wild-type subtilisin E.

An assay for amino acid ligase activity was designed which relied on the synthesis of two substrate monomers which would be joined by subtilisin and subsequently be detectable after ligation. The assay was designed to directly select for ligation of two peptides in chosen concentrations of DMF. The assay takes advantage of the fact that a covalent bond is being formed between the two substrates, therefore allowing unreacted substrate to be removed by washing from a solid support. This concept is depicted in Figure 2. The P1 amino acid (carboxyl donor) is covalently attached *via* its amino terminus to filter paper (structure **1**). The P1' amino acid (amino donor) is covalently labeled with fluorescein at its carboxy terminus (structure **2**). When the carboxyl and amino groups are ligated (forming the peptide bond) the fluorescein label becomes covalently attached to the paper (structure **3**); unligated label can be washed away and recovered. The result is a fluorescent spot on the filter paper corresponding to the position of significant ligase activity. These substrate monomers were successfully synthesized and substrate 1 was attached to nitrocellulose filter paper.

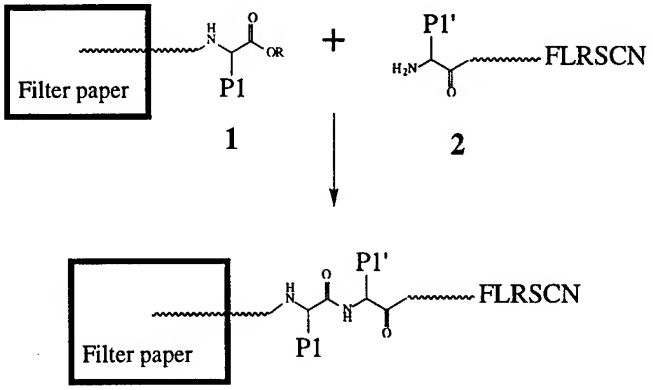


Figure 2 Synthesis of linker substrates

In concept many mutant enzymes could be screened by performing a filter lift assay as depicted in Figure 3. Bacterial colonies expressing subtilisin were grown on rectangular agar plates (**A**). A replica filter lift (**B**) of the colonies was placed on a second piece of filter paper saturated with the substrates **1** and **2**, with **1** being covalently linked to the paper (**C**). The fluorescent substrate **2** was dissolved in the chosen organic solvent (in this case DMF) and this solution was used to saturate the modified filter paper. After incubation (**D**), the cell containing filter lift was removed and the substrate containing filter

paper washed to remove unligated **2**. The position of the covalently attached fluorescein derivative **3** was determined using a standard plate reading fluorometer in gel-scan mode (**E**). A Perkin-Elmer LS50B plate-scanning fluorometer was successfully adapted to a continuous scan mode with 1mm incremental movements in X and Y directions across a 3.75" x 4.25" filter paper. After identification, active colonies from (**A**) were picked and regrown for subsequent rounds of mutagenesis.

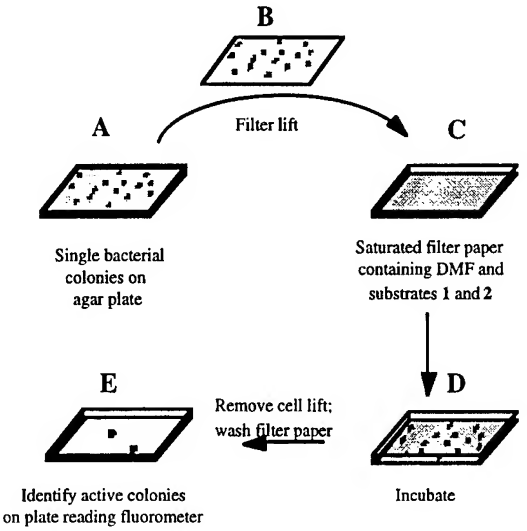


Figure 3 Filter lift screening assay

The assay was put through a validation experiment using wild-type subtilisin E. As a negative control, a strain containing plasmid missing the subtilisin gene was used. The colonies were plated out as in step **A** in Figure 3 and carried through the remaining steps **B-E** using buffers containing no DMF to demonstrate the ability to detect ligase activity in the assay.

The synthesis of **1** and **2** is depicted in Figures 4 and 5. Triethylene glycol can be activated with mesyl chloride followed by displacement with sodium azide to yield the mono and disubstituted derivatives. These can be preparatively separated to yield the precursors for linkers **a** and **b**. Jones oxidation of the azido-alcohol to the azido-acid yields linker **a**; triphenylphosphine monoreduction of the bis azide yields linker **b**. For substrate **1,** linker **a** is coupled to the paper *via* the acid function and reduced to yield the primary amine. Attachment of the amino acid (methionine is used for illustration) is accomplished by first reaction with glutaric anhydride to generate a terminal carboxyl function, and subsequent condensation *via* DCC with linker **a** attached to the paper. For substrate **2** the N-protected amino acid (methionine is again used for illustration) is condensed with linker **b** followed by reduction of the remaining azide and coupling with fluorescein isothiocyanate (FITC), which is selective for amines. Removal of the t-BOC protection group yields the substrate **2.**

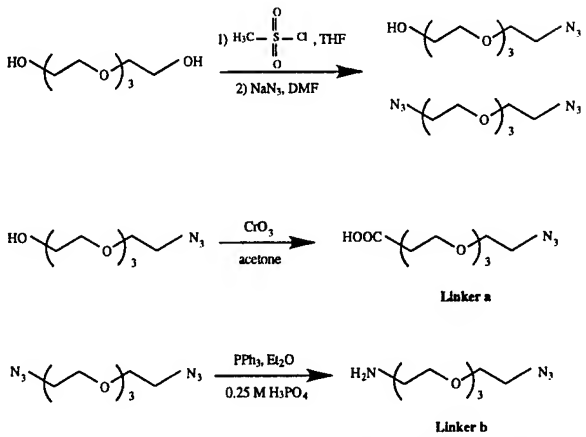


Figure 4. Synthesis of linkers a and b.

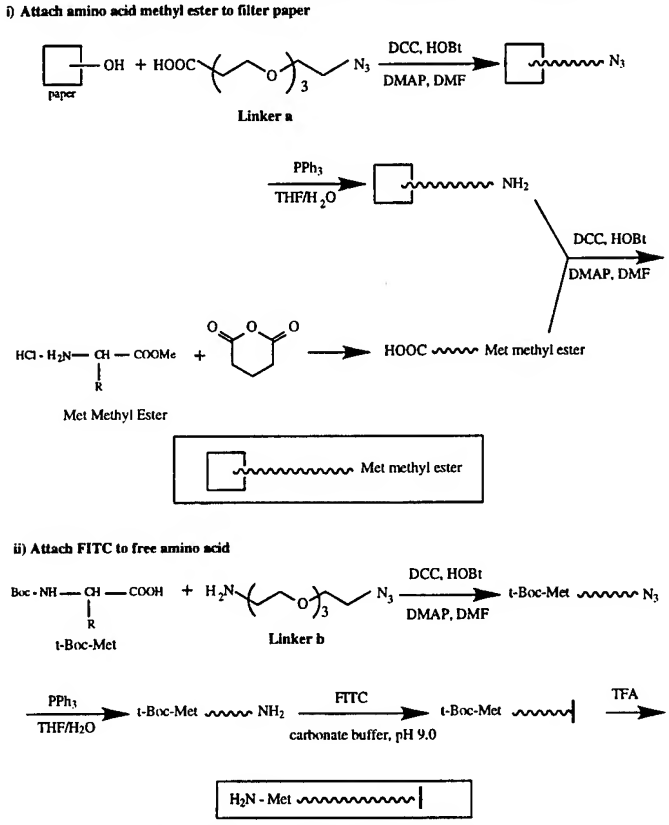


Figure 5. Synthesis of substrates 1 and 2

A major problem with the filter-based assay with attached linkers was encountered during implementation. In brief, the fluorescence background of the filter paper after washing with fluoresceinated linker was too great to allow accurate quantitation of the rates of linkage catalyzed by the enzyme.

Therefore, an alternative assay for screening enhanced ligase activity of subtilisin E in aqueous DMF was developed. This assay was adapted to both petri plates and microtiter dishes.

The petri plate assay was used for initial screening of mutant libraries for enhanced ligase activity. Agar plates (modified Schaefer's medium) were covered with two membranes: first an organic resistant Biodyne nylon membrane, followed by a

nitrocellulose membrane. *Bacillus subtilis* transformants containing the mutant subtilisin library were plated on the nitrocellulose membrane. After overnight incubation at 37°C, the nitrocellulose membrane containing the *B. subtilis* colonies were removed and stored at 4°C. The nylon membrane, containing bound enzyme, was transferred enzyme-side down to an agarose plate containing 10% methionine methyl ester (pH 8-9) in 50% DMF. After incubation at 37°C, turbid spots appeared at locations corresponding to *B. subtilis* colonies. Controls with purified enzyme demonstrate that the size and intensity of the turbid spots is correlated with amount of enzyme activity.

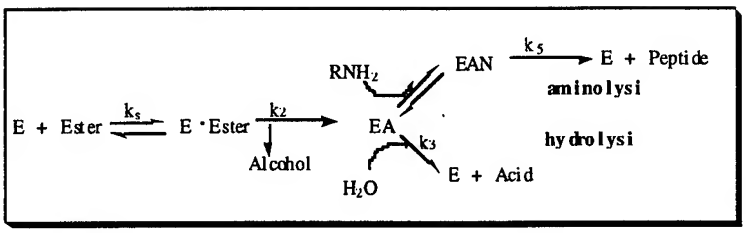
A microtiter dish assay was then developed which was used to better quantitate ligase activity of mutants identified in the petri plate assay. Culture supernatants containing subtilisin from mutants were added to a reaction mix containing methionine methyl ester (pH 8-9) in 50% DMF. Turbidity is quantified in a plate reader by reading at 500nm. The kinetics of formation of the polymer can be followed with this assay by observing the turbidity increase as a function of time. Clones with faster rates of ligation can be identified by observing an increased rate of polymer formation.

The original assay used 10% methionine methyl ester (pH 8-9) in 50% DMF. Addition of the indicator phenol red allowed us to monitor the pH change caused by the hydrolytic activity of the subtilisin during the reaction. This hydrolysis activity competes with the ligase activity of the subtilisin. Briefly, the attack of the acyl-enzyme intermediate by water results in hydrolysis which releases protons, while the attack on the same intermediate by amine results in aminolysis, ie., the addition of a monomer to the growing polymer. Thus the ratio between the rates of aminolysis and hydrolysis is crucial. Desireable mutants are those with higher ratios of aminolysis over hydrolysis.

The color of the solution turns from red to yellow as the reaction progresses. We examined reaction conditions to allow us to monitor both turbidity and color change in the same reaction. At 10% methionine methyl ester, the amount of turbidity that was generated before the pH induced color change was too large to be able to measure both variables. At 1% methionine methyl ester, the pH induced color change occurred but very little turbidity appeared. In addition, 2%, 3%, 4%, 5%, 8% methionine methyl ester were tested with phenol red in the reaction solution. The 2%-5% solutions worked fine, with the color changing first followed by formation of the precipitate. This means that the assay can be done in one microtiter plate. We are currently testing this assay modification in agar plates. This modified assay may be used to identify mutant subtilisins with reduced hydrolysis activity relative to ligase activity.

The absolute rate of peptide synthesis was determined by the following scheme (figure 6). In this scheme the rate of aminolysis is in competition with the rate of hydrolysis. The partition coefficient β expresses the relative direction of the reaction with a large value of β indicating a preference for polymer formation. The partion coefficient may thus be used to as the criterion for choice of the improved mutants.

Protease-Catalyzed Kinetically Controlled Peptide



V_H = rate of hydrolysis = $_3[EA][H_2O]$

$$VA = \text{rate of aminolysis} = \frac{k_5[EA][RNH_2]}{k_N + [RNH_2]} \simeq \frac{k_5}{k_N}\,[EA][RNH_2]$$

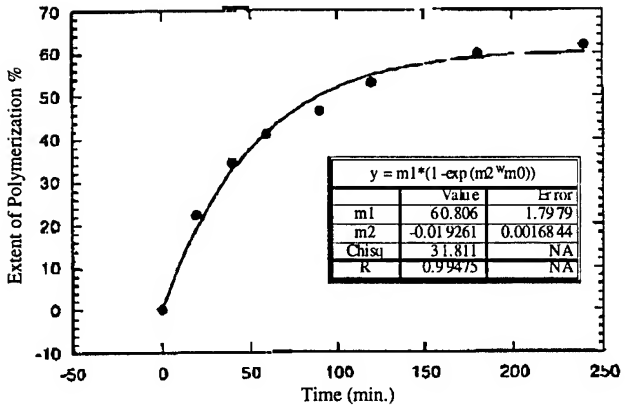
$$\beta = \text{partition constant} = \frac{V_A}{V_H} \times \frac{1}{[N]} = \frac{k_5}{k_N k_3}$$

$$\frac{dp}{dt} = [E]\frac{k_2}{k_s}\,\frac{\beta n s - \alpha p}{1+\beta n} \qquad (1) \qquad \frac{ds}{dt} = -[E]\frac{k_2}{k_s}\,S$$

When $\alpha = \dfrac{k_4/k_5}{k_2/k_s} \longrightarrow 0$ (50% DMF) and $_o \ll n_o$

$$p = \frac{\beta n_0 s_0}{1=\beta n_0}\left\{1 - e^{-[E]\frac{k2}{ks}t}\right\}$$

Time-Course of The Polymerization Rxn (50% DMF)



$$p = \frac{\beta n_0 s_0}{1=\beta n_0}\left(1 - e^{-[E]\frac{k2}{ks}t}\right)$$

Figure 6. Derivation of expressions for kinetic assay of peptide polymerization

2. Mutagenesis

A modified *Bacillus-E. coli* shuttle expression vector was constructed to increase the transformation efficiency of the plasmid in *E. coli.* The shuttle vector was constructed by ligation of two fragments, one from pUB110 and the other from pGEM3. The wild-type subtilisin subtilisin E gene with its promoter was subcloned into this shuttle vector from plasmid pKWZ. The ampicillin gene from *E. coli* vector pGEM3 was introduced into the shuttle vector. This gene expresses well *in E. coli* and its introduction into the shuttle vector increased the transformation efficiency of this plasmid into *E. coli.*

Working together, Edward Bylina of RBI and Huimin Zhao of CalTech performed both error-prone PCR on the wild-type subtilisin E gene and DNA shuffling between the wild-type subtilisin E gene and its mutant 13M gene. Dr. Bylina brought back the necessary vectors, primers and strains from Caltech for mutagenesis at RBI.

The petri dish assay for screening enhanced ligase activity of subtilisin E in aqueous DMF was used to screen for improved mutants. The first round of mutagenesis was performed using 50% DMF and 5% methionine methyl ester. Many positive colonies better than wild-type were observed. In these mutants, the rate of turbidity increase observed in the assay was 2 or 3-fold better than wild-type. After screening approximately 5000 colonies, the five best mutants were picked. The plasmid DNA was isolated from these five mutants and retransformed into DH10B. The inserts in these plasmids were sequenced.

To prepare for the sequencing of mutants, we confirmed our ability to sequence the wild-type subtilisin gene in our plasmid using the primers used for error-prone PCR. Sequence for the entire insert was obtained, but we designed two additional sequencing primers which will allow us to obtain double stranded sequence for each mutant insert.

The transformation efficiency into *Bacillus* was improved to ~10^4 transformants per ug plasmid DNA. Use of the *E. coli* strain HB101 in preparing the mutagenized plasmid library and inclusion of a trace amount of manganese chloride in the culture medium resulted in this efficiency improvement.

3. Sequencing results

The DNA sequences of the subtilisin mutants isolated during the first round of mutagenesis using 50% DMF and 5% methionine methyl ester were determined. The three mutants, those with enhanced rates of synthesis, P20E8, P34B11 and P9D12 have sequence changes as follows:

P20E8
CAA to CAG (Gln 10) silent
ACA to ACT (Thr 22) silent
TCA to TCT (Ser 78) silent
GTT to GAT (Val 143 to Asp)
GGC to TGC (Gly 166 to Cys)
P34B11
TAT to AAT (Tyr 217 to Asn)
P9D12
TCA to CCA (Ser 188 to Pro)
GGC to AGC (Gly166 to Thr)
GCA to GCG (Ala88) silent
CAG to CGG (Gln59 to Arg)

Mutant P9D12 had the highest rate enhancement in DMF. All the amino acid changes in this mutant are located in the loop and clustered around the binding pocket (see figure below). The Ser188 to Pro mutation was previously observed in subtilisin variant 12M, which showed improved hydrolysis in the presence of DMF.

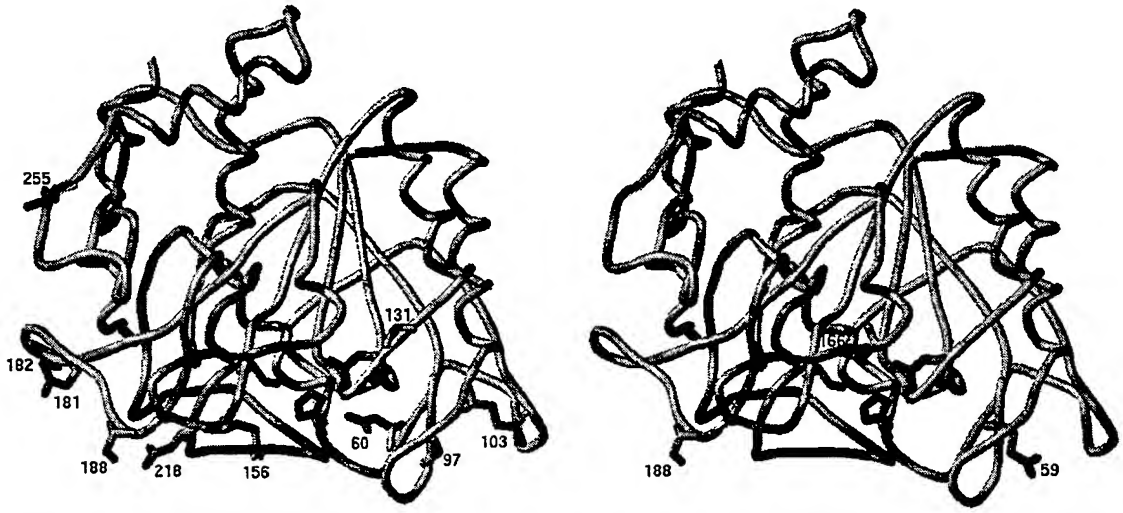


Figure 7. 3-D representation of subtilisin variants 12M (left) and P9D12 (right).

This project has successfully conceptualized assay procedures for amino acid ligation by a peptidase and implemented procedures for the improvement of activity by random mutation of a gene and subsequent screening of expressed enzyme in a polar organic solvent, dimethyl formamide. The subtilisin mutant P9D12 can be used in high concentrations of DMF for synthesis of polyamides. Moreover, the principles discovered in this research are of immediate importance for fields of molecular biology, biochemistry and polymer synthesis.

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